Demonstration of *Chlamydia pneumoniae* in the walls of abdominal aortic aneurysms

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Background: **Seroepidemiologic studies have indicated an association between chronic *Chlamydia pneumoniae* infection and coronary heart disease. The organism, which is a common respiratory pathogen, has been demonstrated in atherosclerotic lesions of the aorta and coronary arteries. Abdominal aortic aneurysms are frequently associated with atherosclerosis, and inflammation may actually be an important factor in aneurysmal dilatation. Hence it could be assumed that *C. pneumoniae* may play a role in maintaining an inflammation and triggering the development of aortic aneurysms.**

Methods and Results: **Specimens from abdominal aortic aneurysm were examined for the presence of *C. pneumoniae* by immunohistochemical analysis, the polymerase chain reaction amplifying ompl gene, transmission electron microscopy, and culture methods with histologically atherosclerosis-negative human aortic tissues used as a control group. Chlamydial lipopolysaccharide and *C. pneumoniae* specific antigens were found by immunohistochemistry in 12 and 8 of 12 aneurysm specimens, respectively, and *C. pneumoniae* DNA could be demonstrated in 6 of 6 aneurysm specimens studied. Furthermore electron microscopy revealed the presence of *Chlamydia*-like elementary bodies in three of four aneurysm specimens tested. None of the control samples gave positive reaction in the polymerase chain reaction, and *C. pneumoniae* antigens were not detected in any of them.**

Conclusions: ***C. pneumoniae*** **is frequently found in the vessel wall of abdominal aortic aneurysm. The potential etiopathogenetic role of *C. pneumoniae* in the development of these aneurysms remains to be studied. (J Vasc Surg 1997;25:499-505.)**

Chlamydia pneumoniae, a recently discovered human pathogen, is a common cause of respiratory infections worldwide.[1,2] It has been estimated that most people have two or three *C. pneumoniae* infections during their lifetime.[1] The prevalence of antibodies to *C. pneumoniae* increases with age and occurs in more than 50% of middle-aged adults in Finland.[2] It is also markedly higher in men than in women in older age groups, a difference that increases with age[2] and is higher in smokers than in nonsmokers.[3-4] It can be estimated that approximately half of the elderly men in Finland have chronic *C. pneumoniae* infection.[5] Recent studies have shown elevated *C. pneumoniae* antibody titers and the presence of immune complexes in most patients with myocardial infarction and coronary heart disease, suggesting chronic infection.[5-9] Furthermore *C. pneumoniae* has been demonstrated in approximately 50% of atherosclerotic lesions from autopsy and atherectomy tissue specimens but not in normal-appearing areas by electron microscopy, immunohistochemistry, and the polymerase chain reaction (PCR), confirming the findings of seroepidemiologic studies.[10,13] Moreover, persistent infection

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Table I. Summary of demographic data, detection of *C. pneumoniae* by immunohistochemistry with monoclonal antibodies specific to clamydial lipopolysaccharide and *C. pneumoniae* proteins, immunoglobulin G antibody titers in serum, in circulating immune complex and bound to tissues, tissue polymerase chain reaction, transmission electron microscopy findings, and isolation results of 12 patients with abdominal aortic aneurysms

Patient no.	*Sex*	*Age (yr)*	*Smoking*	*Immunohistochemistry*		*Immunoglobulin G antibody titer in*			*PCR*	*EM*	*Isolation*
				LPS	*Protein*	*Serum*	*Serum IC*	*Tissue IC*			
1	F	65	no	+++	++	16	<2	2	+	–	–
2	M	76	yes	+++	+	<8	<2	<1	+	NT	–
3	M	65	no	+	–	16	8	4	+	+	–
4	M	67	yes	+++	+	512	4	>4	+	NT	NT
5	M	52	no	+++	+	32	2	NT	+	NT	NT
6	F	71	no	+	+	32	2	NT	+	NT	NT
7	M	64	no	+++	–	NT	NT	NT	NT	+	NT
8	M	73	yes	+++	+	NT	NT	NT	NT	+	NT
9	M	68	yes	++	+	32	2	NT	NT	NT	NT
10	M	67	yes	+++	+	NT	NT	NT	NT	NT	NT
11	M	62	yes	+++	–	NT	NT	NT	NT	NT	NT
12	M	54	yes	+++	–	128	8	NT	NT	NT	NT

LPS, Lipopolysaccharide; *IC,* immune complex; *PCR,* polymerase chain reaction; *EM,* electron microscopy; *NT,* not tested.
*Reactivity graded −/+/++/+++.

and multiplication of *C. pneumoniae* in endothelial cells has been demonstrated in vitro.[14]

Abdominal aortic aneurysms have been associated with atherosclerosis in older patients and are traditionally described as "atherosclerotic."[15] In most cases a conspicuous inflammatory infiltrate is found between the media and adventitia in the aneurysmal wall,[16] and it has been suggested that destruction and weakening of the aortic wall may occur as a result of the release of inflammatory mediators in response to the atherosclerotic process.[17] Inflammation may actually be an important factor in aneurysmal dilatation, although the initial triggers are obscure. Aneurysms of the ascending aorta have been described as a typical consequence of *Treponema pallidum* infection. Once syphilis has almost disappeared, these aneurysms have also became rare. By contrast, abdominal aortic aneurysms have become common in industrialized countries in recent times.[18,19]

Because *C. pneumoniae* has been shown to be present in atherosclerotic lesions and it is capable of multiplying in the cells of the vessel wall, one may surmise that it could be an agent maintaining an inflammation and triggering aortic aneurysm. We report here the frequent presence of *C. pneumoniae* in the walls of abdominal aortic aneurysms by immunohistochemistry, the PCR, and transmission electron microscopy. Further studies are required to elucidate the etiopathogenetic role of *C. pneumoniae* in this disease.

SUBJECTS AND METHODS

Patients and control group. Samples from 12 consecutive patients undergoing the repair of abdominal aortic aneurysms at Oulu University Hospital in spring 1994 were examined. The demographic data of the patients are presented in Table I. The serum samples for serology were obtained before the operation was performed. During surgery a specimen from the anterior wall of the aneurysm sac was obtained for histologic examination and bacterial culture. Slices of the aneurysmal wall were immersed in liquid nitrogen for PCR and *C. pneumoniae* culture and in 10% buffered formalin for immunohistochemistry. Segments of the specimens were also fixed in a glutaraldehyde-formaldehyde mixture for electron microscopy studies. Furthermore a piece of the aneurysmal tissue was also taken for microbiologic analysis by conventional aerobic and anaerobic culture.

Because it was not possible to obtain control samples from living donor tissue, we therefore collected "normal" abdominal aortas from fresh cadavers in the Department of Forensic Medicine (provided by Dr. Terttu Särkioja). We succeeded in obtaining three age-matched male samples from nonarteriosclerotic abdominal aortas of men killed in accidents. All autopsies were performed within 12 hours of death. To control the method and possible errors in autopsy material we obtained punch biopsy specimens from ascending aortas during coronary artery bypass grafting procedures, which were then run at the same time. The same protocol was used for

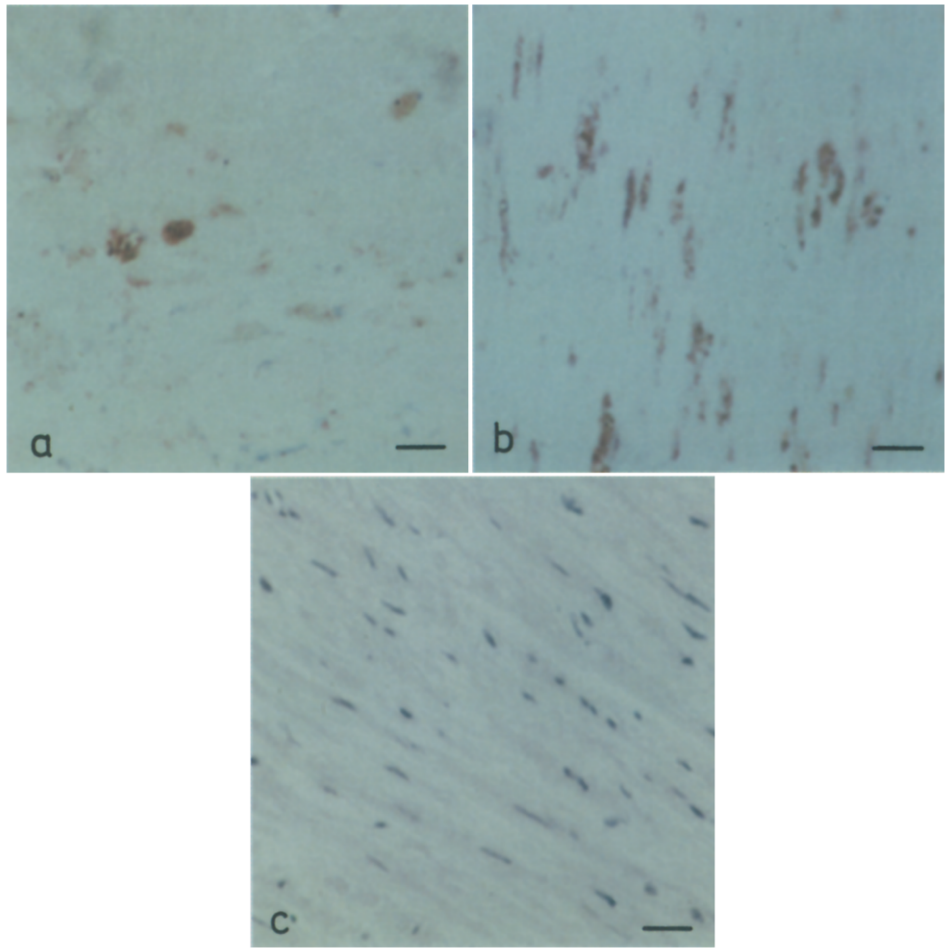

Fig. 1. Immunostaining of aneurysmal aortic wall with *Chlamydia* lipopolysaccharide-specific monoclonal antibody showing strong positive reaction in macrophages of atherosclerotic plaque *(a)* and in smooth muscle cells beneath plaque *(b)*. No staining is seen in normal aorta *(c)*. Bar 50 μm.

all experiments performed on both patient and control aortic material. Six of the control samples were from men, and three were from women (mean age, 63 years; range, 45 to 73 years). Specimens with histologic evidence of atherosclerosis were eliminated from this series. There was not sufficient tissue to run every test for each patient. All data recorded from the patients are reported. In addition, normal skin and uterine wall samples served as negative control tissues.

Immunohistochemistry. The immunohistochemical staining of the formalin-fixed, paraffin-embedded tissues were performed by the avidin/strep-avidin-biotin-peroxidase method of Hsu et al.[20] with the Vectastain ABC kit (Vector laboratories, Burlingame, Calif.) or the Histostain SP kit (Zymed, San Francisco, Calif.). Hematoxylin-eosin was used as the counterstain. A *Chlamydia* group-specific mouse monoclonal antibody against lipopolysaccharide antigen, a species-specific monoclonal antibody against *C. pneumoniae* prepared in our laboratory by Dr. Andrei Kutlin (SUNY, New York, N. Y.), and rabbit polyclonal anti-*C. pneumoniae* were used in the immunostainings. Rabbit antibody against *Francisella tularensis* and normal serum were used as control antibodies for both patient and control samples. *C. pneumoniae*-infected HeLa cells were used as positive control samples, and normal skin and uterine wall samples were used as negative control tissues.

PCR. The tissue samples were treated with proteinase K (Sigma, St. Louis, Mo.) and Nonidet P-40/Tween-20 (Sigma) at final concentrations of 100 μg/ml and 0.5%, respectively. After incubation at 55° C overnight DNA was purified with a QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The

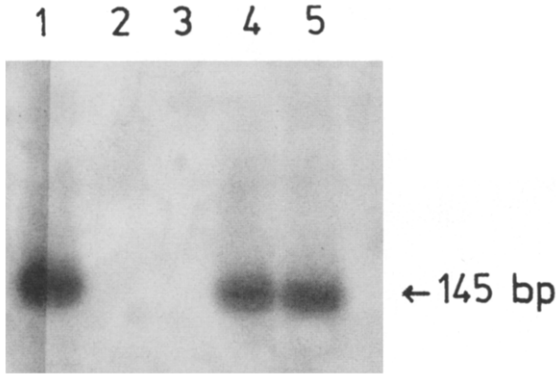

Fig. 2. Southern blotting of polymerase chain reaction products obtained with ompl-specific primers: *Lane 1,* Positive control (DNA extracted from *C. pneumoniae* strain Kajaani 6), *lanes 2* and *3,* two control aortas, and *lanes 3* and *4,* aneurysm tissues of two patients.

oligonucleotide primers used, SC5 (5′TGCCTG-T(AG)GGGAA(TC)CC(AT)(GT)CTGA(AT)CCA3′ and SCH4 (5′GTCGAAAAGA(AT)AGTC(TA)C-C(GA)TAGTA3′), modified from previously published primer sequences[21,22] to be *C. pneumoniae*-specific by Dr. S. Rasmussen (University of California, San Francisco), which amplify a 145 bp sequence at the 5′ end of *C. pneumoniae* ompl gene, were purchased from the Institute of Biotechnology, Helsinki, Finland. The PCR amplification mixture contained 1 mmol/L deoxynucleoside triphosphates, 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.0 U *Taq* polymerase (Hytest, Turku, Finland), 50 pmol of each primer, and 14 μl of processed and diluted sample with total reaction volume of 25 μl. AmpliWax beads (Perkin-Elmer Cetus, Norwalk, Conn.) were used to facilitate the "hot start" PCR.[23] After 5 minutes of denaturation at 94° C the samples were subjected to 40 consecutive cycles of denaturation (94° C, 30 sec), annealing, and extension (60° C, 45 sec) with a Perkin-Elmer Cetus GeneAmp 9600 thermocycler. The final extension time was 6 minutes at 72° C after cycling had been completed. The PCR products were separated by electrophoresis on 3% agarose gels containing ethidium bromide (0.2 μg/ml). DNA was transferred onto a nylon membrane (Immobilon N, Millipore, Bedford, Mass.). The blot was hybridized with a specific digoxigenin-labeled probe (5′CCATAT(TA)CT(GA)CCATCAATTAA3′) in 5× standard saline solution citrate, 1.0% (wt/vol) blocking reagent for nucleic acid hybridization (Boehringer-Mannheim, Mannheim, Germany), 0.1% N-layroylsarcosine, and 0.02% sodium dodecyl sulphate at 43° C. The hybridized probe was detected by alkaline phosphatase-labeled anti-digoxigenin Fab fragments with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Bio-Rad, Hercules, Calif.) as substrate.

Transmission electron microscopy. Specimens for electron microscopy were fixed in a glutaraldehyde-formaldehyde mixture (1% glutaraldehyde, 4% formaldehyde, 0.1 mol/L phosphate buffer, pH 7.4) and postfixed with buffered 1% osmiumtetroxide. Thin sections were cut with Ultracut E microtome (Reichert-Jung) and poststained with uranyl acetate and lead citrate (Carlsberg system, LKB, Sweden). The electron micrographs were taken with a JEOL 1200EX transmission electron microscope operating at 60 kV.

***C. pneumoniae* isolation.** For culturing tissue specimens were homogenized in chlamydia transport medium with a tissue homogenizer and inoculated into HL cell monolayers, a human cell line, in a 24-well flat-bottomed tissue culture plate. Cultures were stained by the fluorescent antibody technique with chlamydia genus-specific monoclonal antibody CF-2.

Measurement of *C. pneumoniae* antibodies. Immunoglobulin class-specific antibodies were measured by the microimmunofluorescence method[5] with elementary bodies of *C. pneumoniae,* strain Kajaani 6, *C. trachomatis,* L2, fluorescein-conjugated anti-human immunoglobulin G (Kallestad, Austin, Texas), immunoglobulin A (Sigma, St. Louis, Mo.), and immunoglobulin M (Dako A/S, Glostrup, Denmark). Immune complexes were precipitated from the serum samples with 3.5% polyethyleneglycol as described earlier.[5] Antibodies to *C. pneumoniae* were measured by microimmunofluorescence from immune complexes dissolved in phosphate-buffered saline solution, pH 7.4 (PBS). For measurement of tissue-bound antibodies a small piece of frozen aneurysmal aortic wall (four samples) was cut into pieces, immersed in a small volume of phosphate-buffered saline solution, and vortexed for 20 minutes. After centrifugation was performed, the supernatant was used for the measurement of antibodies to *C. pneumoniae* antigen possibly present in the tissue.

RESULTS

The demographic data of the patients undergoing surgery for abdominal aortic aneurysms and included in the study is shown in Table I. Ten of 12 patients were men, the age range was 52 to 76 years, and 7 of the patients were smokers.

On histologic evaluation the aneurysmal speci-

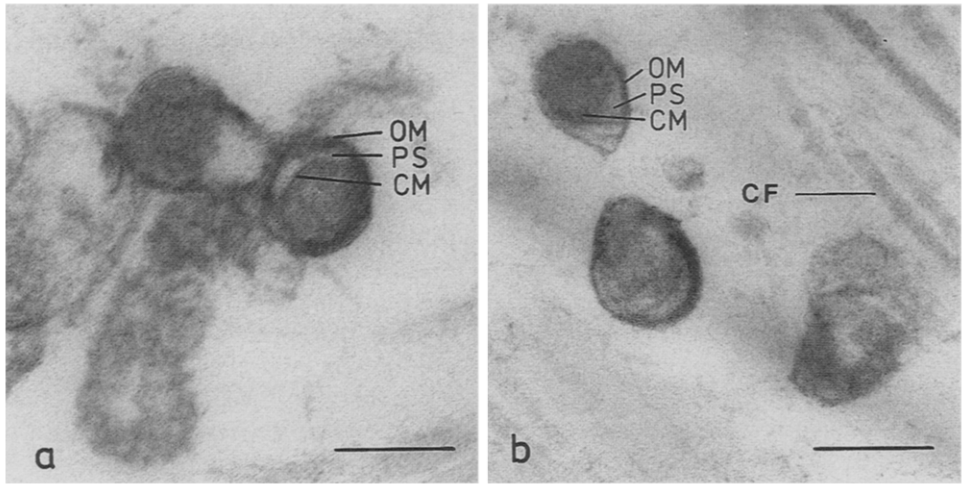

Fig. 3. *Chlamydia*-like particle in vessel wall of abdominal aortic aneurysm of two patients (*a* and *b*). *OM,* Outer membrane; *CM,* cytoplasmic membrane; *PS,* periplasmic space; *CF,* collagen fibers. Bar 0.2 μm.

mens showed severe atherosclerosis with variable inflammatory infiltrates consisting mainly of lymphocytes and plasma cells. Immunohistochemical examination demonstrated chlamydial lipopolysaccharide-antigen in large amounts in the macrophages of the atherosclerotic plaques and in smooth-muscle cells beneath the plaques in all 12 aneurysm specimens (Table I, Fig. 1). Staining with a polyclonal rabbit *C. pneumoniae* antiserum gave a similar picture. The staining with monoclonal *C. pneumoniae*-specific antibody was much weaker than lipopolysaccharide-staining and was observed mainly in macrophages; 4 of the 12 specimens were negative. No positive staining was observed in control specimens obtained from ascending and abdominal aorta nor in the negative control tissues with any of the antibodies used (Fig. 1, *C*). Staining with the antitularaemia or normal rabbit or goat serum in all the specimens was also negative in all the specimens.

The PCR amplification of omp1 gene produced the expected 145 bp band for all six aneurysms tested, indicating the presence of chlamydial DNA (Table I). Control arterial wall specimens were negative for *C. pneumoniae* DNA. The specificity of PCR product was confirmed by Southern hybridization with specific probe (Fig. 2).

Four aneurysm specimens were examined by transmission electron microscopy. Typical *Chlamydia*-like organisms were detected in three of four lesions (Table I). Structures were pear-shaped and had a bilayer cell wall structure (the outer membrane and the cytoplasmic membrane) and a large periplasmic space (Fig. 3). The size (average 0.3 μm) of these organisms was similar to that of cell culture-grown organisms.

Isolation of *C. pneumoniae* was attempted from three aneurysm specimens with negative results. *C. pneumoniae* antibodies were present in seven of eight serum samples tested in titers 16 to 512 (Table I), and circulating *C. pneumoniae*-specific immune complexes were detected in six of eight sera studied. None of the patients had *C. trachomatis* antibodies present in their sera. Patient 2 had neither serum antibodies nor circulating immune complexes present. Aneurysmal tissues from the first four patients (Table I) were also tested for the presence of tissue-bound antibodies, and in three of them bound antibodies could be demonstrated. Aerobic and anaerobic bacterial cultures from the specimens were negative in every instance.

DISCUSSION

C. pneumoniae is an obligatory intracellular bacterium that is able to multiply in monocytes, alveolar macrophages,[24] and vascular endothelial cells in vitro.[14] Furthermore it can cause a persistent infection in cultured endothelial cells.[14] In this study we demonstrated the presence of chlamydial components in macrophages located in abdominal aortic aneurysm lesions and in smooth muscle cells beneath the plaques. This finding is very similar to that earlier demonstrated in atherosclerotic lesions,[10,12] except that in those cases only approximately half of the lesions obtained at autopsy or from atherectomy tissues were positive for *C. pneumoniae* when electron microscopy, immunohistology, and PCR were used,

and often only one of the methods gave a positive finding. In this series all the aneurysms were positive by immunohistology and all six specimens studied by PCR, suggesting high numbers of *C. pneumoniae* in the samples and possible active infection in the lesion. Furthermore we were able to demonstrate pear-shaped *C. pneumoniae*-like organisms[10] in three of four aneurysm tissues examined by transmission electron microscopy. The inability to culture the organism from the aneurysm tissue was not unexpected; *C. pneumoniae* has also been found in previous studies to be difficult to isolate.[10]

We demonstrated *C. pneumoniae* in the aneurysms by immunostaining with a *Chlamydia*-genus-specific antibody reacting with the lipopolysaccharide common to all chlamydial species, suggesting that chlamydial lipopolysaccharide is present in high amounts in this infection. The primers used in the PCR products in this study did not amplify DNA of *C. trachomatis* (data not shown). Furthermore we have used the same primers for detection of *C. pneumoniae* from different mouse tissues in our animal studies very successfully without false-positive findings from uninfected animals. The reactivity of most specimens with a monoclonal antibody recognizing only *C. pneumoniae* confirms that our findings are specific to *C. pneumoniae.* In addition, none of the patients had antibodies to *C. trachomatis* present in their sera. The presence of antibodies to *C. psittaci,* which is rare in Finland, was not excluded.

Despite the direct evidence on the presence of *C. pneumoniae* in all 12 patients with abdominal aortic aneurysms, one patient had no *C. pneumoniae* antibodies, and two had only low titers present in the sera. A similar finding was reported earlier; only 60% of the autopsy sera obtained from persons with atherosclerotic lesions positive for *C. pneumoniae* contained demonstrable antibodies to *C. pneumoniae.*[25] One explanation for this result could be binding and sequestration of the antibodies to *C. pneumoniae* antigens present in the circulation as immune complexes or in the tissues of the arterial walls. We were able to show here that one patient with a low-antibody titer did have *C. pneumoniae* antibodies present in circulating immune complexes, and we were also able to dissociate *C. pneumoniae* antibodies from aneurysmal tissue in three of four patients, although blood contamination could not be excluded in the case of those with circulating antibodies.

The pathogenesis of abdominal aortic aneurysms is largely unknown. Degradation of elastin and collagens in the aortic wall has been shown to impair its compliance and thus may lead to aneurysmal dilatation.[26] Increased proteolysis has been shown to play an important role in this process.[27] Inflammation has been shown to mediate connective tissue proteolysis, as best characterized in pulmonary inflammation associated with emphysema.[28] Although the initiator is not known, it is possible that the massive presence of *C. pneumoniae* in these lesions could provoke and feed this inflammation. Chronic chlamydial infections, although usually scarring and occluding,[29] also contain an inflammatory component that may participate in the formation of aneurysms. Interferon γ, produced in intracellular infections, is thought to be a key factor in inducing persistent chlamydial infections[29] and is present in chronic chlamydial lesions.[30] It is known to induce oxidative processes, to reduce synthesis of collagen, to lead to activation of elastase, and to inhibit the proliferation of vascular smooth muscle[31] and endothelial cells.[32] A bacterial protease has been described for *C. psittaci,*[33] and our preliminary studies suggest that *C. pneumoniae* either produces itself or induces the production of several proteases when growing in the cell cultures (K. Valkonen et al., unpublished data). Smoking is one major risk factor for abdominal aortic aneurysm, and stopping smoking has recently been found to improve the prognosis for the disease.[34] The mechanism by which smoking affects the aneurysmal wall is unknown, but several authors have associated smoking with chronic *C. pneumoniae* infections.[3,4] It is tempting to speculate that the promotion of a chronic *C. pneumoniae* infection in the lungs by smoking could also lead to infection of the aneurysmal walls by the same agent. Chronic obstructive pulmonary disease recently associated with *C. pneumoniae* infection[35,36] is a major risk factor for aneurysms.[37] Because antibiotics that are effective against *C. pneumoniae* exist, intervention trials would be a logical next step to confirm the association between *C. pneumoniae* infection and abdominal aortic aneurysms.

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